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LIPID-LADEN MACROPHAGES DOWNREGULATE AKT
PHOSPHORYLATION AND METABOLIZE LIPID DROPLETS VIA AUTOPHAGY

By

RIFAT SULTANA

A thesis submitted in partial fulfillment of the requirements for the

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LIPID-LADEN MACROPHAGES DOWNREGULATE AKT PHOSPHORYLATION
AND METABOLIZE LIPID DROPLETS VIA AUTOPHAGY

This thesis is approved as a creditable and independent investigation by a
candidate for the Masters of Science in Biological sciences degree and is acceptable for
meeting the
thesis requirements for this degree. Acceptance of this thesis does not imply that the
conclusions reached by the candidate are necessarily the conclusions of the major
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This thesis is dedicated to my thesis advisor and my lab mates for their friendly support and guidance, my parents, and my daughter for helping me survive all the stress during this year and not letting me give up.

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CONTENTS

ABBREVIATIONS.....	VI
ABSTRACT.....	IX
CHAPTER 1.....	1
LITERATURE REVIEW.....	1
INTRODUCTION.....	1
FOAM CELLS IN ATHEROSCLEROSIS.....	2
LIPID DROPLETS.....	5
AUTOPHAGY.....	6
CONCLUSION.....	9
REFERENCES.....	10
CHAPTER 2.....	15
LIPID LADEN MACROPHAGES DOWNREGULATE AKT PHOSPHORYLATION AND METABOLIZE LIPID DROPLETS VIA AUTOPHAGY.....	15
INTRODUCTION.....	15
MATERIALS AND METHODS.....	17
RESULTS.....	22
DISCUSSION.....	28
REFERENCES.....	30
CHAPTER 3.....	47
CONCLUSIONS.....	46
REFERENCES.....	49

ABBREVIATIONS

LDL	Low density lipoprotein
APOB	Apolipoprotein B
LDLR	Low density lipoprotein receptor
PRR	Pattern recognition receptor
IgG	Immunoglobulin G
SRA	Scavenger receptor A
MARCO	Macrophage receptor with collagenous receptor
CD36	Cluster of differentiation
SRB1	Scavenger receptor Class B member 1
LOX1	Lectin type oxidized low density lipoprotein 1
SREC1	Scavenger receptor Class C member 1
SRPSOX	Scavenger receptor for phosphatidylserine and oxidized low-density lipoprotein
CXCL16	Chemokine (C-X-C motif) ligand 16
FC	Free Cholesterol
CE	Cholesteryl ester

ACAT	Acyl Co-enzyme A: cholesterol acyltransferase 1
ER	Endoplasmic reticulum
nCEH	Neutral cholesterol ester hydrolase
RCT	Reverse cholesterol transport
HDL	High density lipoprotein
ABCA1	ATP-binding cassette transporter A family member 1
ABCG1	ATP Binding Cassette Subfamily G Member 1
ApoA1	Apolipoprotein A1
DGAT2	Acyl-CoA: diacylglycerol acyltransferase 2
Rab	small GTPase
TG	Triglycerides
PAT	Perilipin, Adipophilin, TIP4
PLIN1	Perilipin 1
PLIN2	Perilipin 2
PLIN3	Perilipin 3
PLIN4	Perilipin 4
PLIN5	Perilipin 5
Vps34	Phosphatidylinositol 3 Kinase for cytoplasm to vacuole transport
Vps15	Ubiquitin binding Serine/Threonine Kinase
Atg3	Autophagy related protein 3
Atg4	Autophagy related protein 4
Atg5	Autophagy related protein 5

Atg7	Autophagy related protein 7
Atg9	Autophagy related protein 9
Atg10	Autophagy related protein 10
Atg12	Autophagy related protein 12
Atg16	Autophagy related protein 16
LC3	Light chain 3
PE	Phosphatidylethanolamine
LC3I	Cytosolic form of light chain 3
LC3II	Phosphatidylethanolamine conjugated light chain 3
3MA	3 methyl adenines
GFP	Green fluorescent protein
Akt	Protein kinase B
pAkt	Phosphorylated protein kinase B
ULK1	Serine/Threonine kinase
mTOR	Mammalian target of rapamycin
BMM	Bone marrow media
FBS	Fetal bovine serum
DMEM	Dulbecco's modification of Eagles Medium
DPBS	Dulbecco's phosphate-buffered saline
Ac-LDL	Acetylated low density lipoprotein
PVDF	Polyvinylidene fluoride
PFA	Paraformaldehyde

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ABSTRACT

Macrophages contribute to plaque formation in atherosclerosis. Macrophages take up modified low-density lipoproteins and store excess cholesterol and triglycerides in lipid droplet organelles. Evidence of lipid-laden macrophages or “foam cells” is apparent on histology sections of diseased arteries, and this lipid-laden appearance can be recreated in cell culture upon exposure of cultured macrophages to acetylated LDL (Ac-LDL). Under nutrient stress, neutral lipids in lipid droplets are hydrolyzed by lipolysis, autophagy, or both. However, these processes are not well understood in macrophages. We created lipid-laden macrophages by 24-h exposure to Ac-LDL and analyzed dynamics of lipid droplet metabolism following removal of Ac-LDL from cell culture medium. We found that lipid droplets in these macrophages are cleared within 24 h of Ac-LDL removal from the medium. We then analyzed co-localization of adipophilin, a lipid droplet marker, and LC3, an autophagy marker, with lipid droplets. Results from induction of autophagy by rapamycin, and inhibition of autophagosome formation by 3-methyl adenine suggests the involvement of autophagy in lipid droplet disappearance from cells in the absence of Ac-LDL in the culture media. Studying the mechanisms of lipid droplet degradation will allow us to understand how lipid droplets could be cleared from foam cells resident in atherosclerotic plaques and identify potential therapeutic targets for treating atherosclerosis.

CHAPTER ONE

LITERATURE REVIEW

INTRODUCTION

Heart attack is the leading cause of death in U.S. and kills more than 600,000 people each year (Benjamin, Blaha et al. 2017). The pathological condition responsible for heart attack is known as atherosclerosis. The formation of plaque in the arterial walls causes constriction of blood vessels. Rupture of the plaque and subsequent clot formation blocks the flow of oxygen-rich blood and starves the heart muscle of oxygen, causing “heart attack.”

Lipid-laden macrophages or foam cells are the hallmark of atherosclerosis (Stary, Chandler et al. 1994, Bobryshev 2006). Increased uptake of modified low-density lipoprotein (LDL) and/or reduced cholesterol efflux leads to the formation of intracellular organelles called lipid droplets. Macrophages with excessive lipid droplets are known as foam cells (Yu, Fu et al. 2013). These cells contribute to the plaque formation in atherosclerosis (Ross 1999). Lipolysis and lipophagy (Kovsan, Bashan et al. 2010) are the two known mechanisms for metabolism of lipid droplets. However, lipid droplet metabolism in macrophages is not clearly understood. Understanding the mechanisms of degradation of lipid droplets in macrophages is important in to find therapeutic target for the treatment of atherosclerosis.

FOAM CELLS IN ATHEROSCLEROSIS

Monocyte recruitment

Macrophages play a key role in the initiation and progression of atherosclerosis. Recruitment of monocytes to the intima of the artery wall and differentiation into macrophages is the early stage of plaque formation in atherosclerosis. Uncontrolled uptake of modified low-density lipoprotein (LDL) by macrophages and defective cholesterol transport leads to the formation of lipid droplets and subsequent formation of foam cells (Feng and Tabas 2002).

Cholesterol uptake

Cholesterol uptake in the form of modified low-density lipoprotein (LDL) is considered the earliest stage of foam cell formation. Macrophages can take up apolipoprotein B (APOB) containing lipoproteins via LDL receptors (LDLR) (Moore, Sheedy et al. 2013). However, during early stages of foam cell formation those receptors get downregulated in response to increased intracellular cholesterol levels. This gave rise to the idea that LDLs might be modified in some ways and endocytosed by macrophages through other non-LDLR mechanisms.

Chemical modifications to LDL include oxidation and acetylation and lead to alternative routes of uptake by macrophages. Increased oxidative stress leads to LDL modifications that generate damaged signals which recognized by pattern recognition receptors on immune cells (Moore, Sheedy et al. 2013). This idea came from work in which oxidized epitopes of LDLs were detected in human and mouse atheroma by natural antibodies (Miller, Choi et al. 2011). In the arterial wall, LDLs can also be oxidized by

enzymes such as 12/15-lipoxygenase, myeloperoxidase, and free radicals such as superoxide, hydrogen peroxide, nitric oxide (Miller, Choi et al. 2011).

Macrophages express scavenger receptors such as pattern recognition receptors which can recognize and process modified LDLs and take part in atherosclerosis (Moore and Freeman 2006). This scavenger receptor family includes scavenger receptor A (SRA), macrophage receptor with collagenous receptor (MARCO), cluster of differentiation 36 (CD36) (Sun, Boyanovsky et al. 2007), scavenger receptor class B member 1 (SRB1) (Sun, Boyanovsky et al. 2007), lectin-type oxidized low-density lipoprotein receptor 1 (LOX1) (Chen, Masaki et al. 2002), scavenger receptor class E member 1 (SREC1) and scavenger receptor for phosphatidylserine and oxidized low-density lipoprotein (Shimaoka, Kume et al. 2000) which are responsible for binding to oxidized LDL and formation of foam cells. Among these receptors, scavenger receptor A (SRA) and CD36 are responsible for 75-90% degradation of acetylated or oxidized LDLs *in vitro* (Kunjathoor, Febbraio et al. 2002). These receptors internalize modified low-density lipoproteins by receptor mediated endocytosis.

Cholesterol esterification and re-esterification cycle

The balance of free cholesterol and cholesterol esters is critical for overall cholesterol content in macrophage foam cells. After internalization of modified LDLs, macrophages process those in late endosomes or lysosomes (Goldstein, Dana et al. 1975). This is the organelle where lysosomal acid lipases hydrolyze cholesterol esters to produce free cholesterol (Goldstein, Dana et al. 1975). Free cholesterol is toxic for cells. To prevent cell toxicity, released free cholesterol from lysosomes are re-esterified in

endoplasmic reticulum by acyl co-enzyme A: cholesterol acyltransferase 1 (ACAT 1). Re-esterified cholesterols are then stored in cytoplasmic lipid droplets (Chistiakov, Bobryshev et al. 2016). The re-esterification process by ACAT1 is opposed by neutral cholesterol ester hydrolase which hydrolyze cholesterol esters for efflux out of cells. When ACAT1 activity dominates over neutral cholesterol esters activity, macrophages get filled up with cholesterol esters and turn into foam cells.

Cholesterol efflux

Cholesterol efflux is the first step of reverse cholesterol transport. Macrophages can respond to cholesterol ester accumulation by passing cholesterol through transporters or passive diffusion to extracellular high-density lipoprotein (HDL) or apolipoprotein A1. Several transporters known as ATP-binding cassette transporter A family member 1 (ABCA1), ATP-binding cassette transporter A family member 1 (ABCG1) and scavenger receptor B family member 1 (SRB1) help facilitate cholesterol efflux from macrophages. *In vivo* study of atherosclerosis in mice revealed that expression of both ABCA1 and ABCG1 are important for cholesterol efflux and preventing foam cell formation (Yvan-Charvet, Ranalletta et al. 2007). ATP binding cassette transporter A1 associates with apolipoprotein A1 to facilitate cholesterol efflux out of cells (Wang, Silver et al. 2000). Whereas, ATP binding cassette transporter G1 transports cholesterol to high density lipoprotein (Wang, Lan et al. 2004).

LIPID DROPLETS

Structure & composition of lipid droplets

Lipid droplets are round structures of 0.1-5 μm in diameter in non-adipocytes and can be up to 100 μm in white adipocytes (Fujimoto and Parton 2011). Lipid droplets are composed of neutral lipids such as triglycerides, cholesterol and cholesteryl esters surrounded by a phospholipid mono-layer (Tauchi-Sato, Ozeki et al. 2002, Bartz, Li et al. 2007). The membrane of lipid droplets contains numerous different proteins, of which the majority are structural proteins in the perilipin family (Brasaemle 2007). Some lipid synthesis enzymes such as acetyl coenzyme A (CoA) carboxylase, acyl-CoA synthetase and acyl-CoA:diacylglycerol acyltransferase 2 (DGAT2) (Kuverschner, Moessinger et al. 2008); lipases such as adipose tissue triacylglycerol lipase; and membrane-trafficking proteins like small GTPase Rab family proteins are also found on lipid droplets (Kuverschner, Moessinger et al. 2008). Based on the composition, intracellular lipid droplets are predominantly divided into triglyceride-rich lipid droplets and cholesteryl ester-rich lipid droplets in mammalian cells. Different compositions of lipid droplets depend on the tissue that accumulates lipid droplets and the metabolic function of that tissue (Khor, Ahrends et al. 2014). In adipocytes, lipid droplets are predominantly composed of triglycerides, but in most cells types both triglycerides and cholesteryl esters coexist in various ratios. Even though no demarcation is found in the lipid droplet core under electron microscope, in some circumstances at least triglycerides and cholesterol esters may be segregated from each other (Czabany, Wagner et al. 2008, Cheng, Fujita et al. 2009).

Lipid droplet associated proteins

Lipid droplet associated proteins are known as PAT proteins and consist of perilipin, adipophilin and TIP4 proteins. In mammals, the PAT family has five members: perilipin 1 (PLIN1), perilipin 2 (PLIN2/adipophilin/adipose differentiation-related protein/ADFP), perilipin 3 (PLIN3/Tip47), perilipin 4 (PLIN4/S3-12), and perilipin 5 (PLIN5/lipid storage droplet protein 5/myocardial lipid droplet protein/OXPAT/PAT1) (Bickel, Tansey et al. 2009). Different cell types have different lipid droplets. Different types of lipid droplets are distinguished based on protein composition. Even in the same cell, different sized lipid droplets have different proteins on them. In macrophage foam cells, perilipin 2 or adipophilin are predominantly associated with lipid droplets (Paul, Chang et al. 2008).

AUTOPHAGY

Autophagy is an ancient process that plays an important role in the degradation of cellular organelles. Under starvation condition, it delivers cellular constituents to the lytic compartments of cells. Through this process, cells can degrade damaged organelles as well as serve to combat infection by different kinds of pathogens (Swanson, Byrne et al. 2009). There are three kinds of autophagy: 1) chaperone-mediated autophagy in which molecular chaperones recognizes single proteins and delivers them to lysosomes for degradation; 2) micro-autophagy in which lysosomes internalize cellular constituents by invaginating and pinching off lysosomal membranes to the core; and 3) macro-autophagy, where a de-novo organelle named auto-phagosomes are formed surrounding damaged organelles (Ravikumar, Futter et al. 2009). Those auto-phagosomes then fuse with lysosomes to deliver damaged organelles for degradation (Singh and Cuervo 2011).

Macro-autophagy

As described above, autophagosomes are formed in macroautophagy to deliver degradative organelles to lysosomes. Macroautophagy is a multistep process. It starts from phagophore initiation and ends through the degradation of the organelle.

Initiation of auto-phagosome formation

In mammals, macroautophagy initiates with the formation of autophagosomes. Autophagosome formation is associated with the development of a kinase complex that is formed by beclin, Vps34, and Vps15 and different modulatory proteins to the organelle membranes. The kinase complex together with the delivery of lipids and continuous shuttling of Atg9 forms isolation membranes. The isolation membrane is also called the phagophore. The phagophore membrane mostly originates from the mitochondria (Hailey, Rambold et al. 2010), endoplasmic reticulum (Hayashi-Nishino, Fujita et al. 2009), trans-Golgi network (Tooze and Yoshimori 2010), late endosomes (Longatti and Tooze 2012), and plasma membrane (Ravikumar, Moreau et al. 2010). Elongation of the phagophore is an important stage of autophagosome formation. Two ubiquitin-like conjugation systems such as the Atg12-Atg5-Atg16 system and microtubule-associated protein 1 light chain 3 (LC3) system or Atg8 are required for the elongation and maturation of autophagosomes (Kaufmann, Beier et al. 2014). In Atg12-Atg5-Atg16 system, Atg12 is first evolved by Atg7 in an ATP-dependent mechanism. Atg12 is then transferred to Atg7 to form an intermediate complex Atg12-Atg10. After being covalently attached to Atg5, Atg12-Atg5 complex further interacts with Atg16 homodimer to produce Atg12-Atg5-Atg16 complex. In the second ubiquitin-like conjugation system, phosphatidylethanolamine is conjugated

to Atg8/microtubule-associated protein 1 light chain 3 (LC3). Phosphatidylethanolamine-conjugated LC3 is further processed by Atg4, Atg7 and Atg3 (Geng and Klionsky 2008, Nakatogawa 2013) for the transformation of LC3I to LC3II. Hence, LC3II is accepted as a marker of autophagy (Glick, Barth et al. 2010). Autophagy is also responsible for the cholesterol efflux in macrophages. Knocking down of autophagy related gene decreases autophagy efflux (Ouimet, Franklin et al. 2011).

Fusion of auto-phagosomes with lysosomes

The last step of autophagy is characterized by the fusion of autophagosomes with lysosomes to form autolysosomes and their subsequent degradation (Mizushima 2007). It is also proposed that autophagosomes fuse with endosomes (Liou, Geuze et al. 1997) or late endosomes (Hyttinen, Niittykoski et al. 2013) prior to fusion with lysosomes. Both deliver cargo and the components necessary for membrane fusion machinery, and lower the pH of autophagic vesicle. Autophagosomes that are fused with lysosomes are called autolysosomes. This fusion is regulated by lysosomal membrane proteins Lamp1 and Lamp2 (Tanaka, Guhde et al. 2000). In autolysosomes, the autophagic lumen is degraded by the infusion of hydrolases and acidification through the action of proton pump provided by lysosomes (Ishida, Nayak et al. 2013).

Autophagy is essential for the breakdown of lipid droplets in hepatocytes (Singh, Kaushik et al. 2009). Triglyceride synthesis was measured after knocking out autophagy related gene Atg5 by small inhibitory RNA technique in mouse embryonic fibroblast as an indication of lipid droplet formation and free fatty acid β -oxidation. There was an equivalent increase in the triglyceride synthesis in control and siATG5 cells after treatment

with oleate. The rate of β -oxidation increased after lipid loading, but it decreased after further inhibition of autophagy by 3-methyladenine (3MA). TG breakdown was also greater in control cells than in siATG5 cells (Singh, Kaushik et al. 2009). These results suggest that autophagy is required for lipid droplet degradation in fibroblasts.

Autophagic machinery interacts with lipid droplets during lipophagy (Singh, Kaushik et al. 2009). A small GTPase, Rab7, mediates the docking of autophagosomes, multi-vesicular bodies and lysosomes during lipophagy in hepatocytes (Schroeder, Schulze et al. 2015). To mimic nutrient starvation induced activation of Rab7, a mutant GFP-tagged Rab7 was overexpressed in cells. Increased association of multi-vesicular bodies and lysosomes with lipid droplets was observed which was greater than the wild type GFP-tagged Rab7 (Schroeder, Schulze et al. 2015).

CONCLUSION

Cholesterol burden in macrophages is a major risk factor in atherosclerosis. The idea of autophagy has recently come into light in removing cholesterol through degradation of lipid droplets from macrophage foam cells. Understanding this process will help to identify effective therapeutic targets for treatment of atherosclerosis. This research is aimed at understanding mechanisms of autophagy in lipid-laden macrophages and investigating if there is any connection between autophagy and lipid droplet degradation or cholesterol efflux from foam cells.

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CHAPTER TWO

LIPID-LADEN MACROPHAGES DOWNREGULATE AKT PHOSPHORYLATION AND METABOLIZE LIPID DROPLETS VIA AUTOPHAGY

INTRODUCTION

Plaque formation in atherosclerosis causes cardiovascular diseases like heart attack, stroke and peripheral artery disease. Heart disease is the leading cause of death in USA where it kills more than 600,000 people each year (Benjamin, Blaha et al. 2017). Hence, atherosclerosis has a profound impact on public health. At present, lowering blood cholesterol levels is the primary therapeutic strategy for treating heart disease. However, currently available therapeutics prevent only 30% of clinical events (Klingenberg and Hansson 2009). The high mortality rate due to heart diseases demands investigation of the cellular nature of the disease process to enable discovery of novel drug targets.

The primary stage of plaque initiation is the formation of macrophage foam cells. Macrophages internalize modified low-density lipoproteins through scavenger receptors and by other mechanisms (Brown, Goldstein et al. 1979). When viewed by microscope, atherosclerotic plaque macrophages are found to be filled up with lipid droplets and are called foam cells due to their appearance. Foam cell formation leads to the production of inflammatory cytokines, proliferation and migration of smooth muscle cells and

narrowing of the artery wall (Buckley and Ramji 2015). After macrophages get filled up with lipid droplets, their ability to phagocytose dead and dying cells diminishes (Szondy, Garabuczi et al. 2014). The presence of dead cells destabilizes the plaque leading to plaque rupture and thrombosis. Thus, targeting mechanisms to clear lipid droplets from macrophages may be an effective way to treat atherosclerosis.

Recent work shows that autophagy and macro-autophagy promote lipid droplet cholesterol efflux from macrophage foam cells (Ouimet, Franklin et al. 2011). Electron microscopy characterization of the cholesterol ester cycle in murine macrophage foam cells shows that 20% of lipid droplets are associated with specialized double membrane structures characteristic of nascent autophagosomes (McGookey and Anderson 1983). In another study describing cholesteryl ester accumulation in macrophages, some lipid droplets were surrounded by classical single phospholipid bilayered membranes which indicates the existence of lipid droplets sequestered inside of lysosomes (Brown, Ho et al. 1980). Since hydrolysis of cholesteryl esters occurs both in the cytoplasm and lysosomes, these previous studies raise the question if autophagy delivers lipid droplets to lysosomes for hydrolysis of cholesterol esters and contributes to the reverse cholesterol transport process (Avart, Bernard et al. 1999).

Autophagy is activated in cells through the inhibition of the mammalian target of rapamycin (mTOR) complex which is regulated by protein kinase B (Akt). The activated form of Akt (pAkt) leads to the activation of mTOR which in turn phosphorylates and inhibits ULK1 (Hahn-Windgassen, Nogueira et al. 2005, Nazio, Strappazzon et al. 2013). Activated ULK1 induces autophagy (Russell, Tian et al. 2013) so Akt activation ultimately leads to the inhibition of autophagy.

Ablation of autophagy by knocking out autophagy related gene Atg5 impairs the delivery of lipid droplets to lysosomes for lysosomal acid lipase mediated cholesteryl ester hydrolysis in foam cells (Ouimet, Franklin et al. 2011). In contrast, autophagy is found to deposit fatty acids in lipid droplets in fibroblast cells (Rambold, Cohen et al. 2015). Now the questions are: What is the role of autophagy in lipid droplet metabolism? How do macrophages signal for autophagy of lipid droplets? And, what mechanisms facilitate autophagy machinery to selectively surround lipid droplets to form autophagosomes? Here, I test the hypothesis that formation of lipid droplets activates autophagy in macrophages through the inhibition of Akt and its downstream signaling pathway.

MATERIALS AND METHODS

Medium and reagents

Primary murine macrophages were differentiated and grown in bone marrow medium (BMM). Bone marrow medium contains 20% premium inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA), 30% L-cell conditioned medium (Willert, Brown et al. 2003), 10,000 I.U penicillin and 10 mg/mL streptomycin (Corning, Manassas, VA), 5.7 mM 2-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA), and Dulbecco's Modified Eagles Medium (DMEM) containing 4.5 g/L glucose, 0.5 g/L L-glutamine and 0.1 g/L sodium pyruvate (Corning, Manassas, VA). All these materials were mixed together and filtered through a 0.22 μ m filter (EMD Millipore, Darmstadt, Germany).

Bone marrow isolation from femur of mouse

Adult black 6 (C57BLJ) mice were received from Jackson Laboratories (Bar Harbor, ME). Mice were euthanized using CO₂ and femurs were harvested from the mice carefully so that the ends of the femur remained intact (Swanson 1989). The ends of the femur were cut off and bone marrow was flushed using DPBS without calcium or magnesium (GE Healthcare Life Sciences, Pittsburgh, PA) using a 0.5 inch, 26-gauge needle and 5 ml sterile Luer Lock syringe. Bone marrow was centrifuged at 800 g for 10 min. Cells were resuspended in BMM and plated at a density of 10 million cells in non-tissue culture treated, sterile, 10 cm petri dishes with 10 ml of BMM. The plates were then incubated in an incubator at 37°C provided with 5% CO₂. Two days later an additional 10 ml of BMM was added to the dish. On day 4 following isolation, non-adherent cells were discarded and adherent cells were lifted from the dishes using cold DPBS without calcium or magnesium and frozen in BMM containing 10% dimethyl sulfoxide at a concentration of 1×10^6 cells/ml. Following a slow freeze at -80° F, cells were stored in liquid nitrogen until use. For experiments, cell vials were taken from nitrogen tank, thawed, and resuspended in bone marrow medium.

Formation of lipid-laden macrophages

Bone marrow macrophages were plated in non-tissue culture treated 24-well plates and incubated for 2 h for attachment to the surface of coverslips. Cells were

exposed to 50 µg/ml acetylated low density lipoprotein (Ac-LDL, Alfa Aesar, Haverhill, MA) for 24 h. During this period, lipid droplets were observed to form in macrophages.

Immunofluorescence Experiment

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde for 30 min, permeabilized and blocked with 2.5% bovine serum albumin 1 h. After overnight incubation with primary antibodies (anti-LC3 and anti-adipophilin from Abcam, Cambridge, MA, USA) cells were incubated with secondary antibodies (anti-Rabbit IgG, DyLight488 conjugate, Invitrogen, Carlsbad, CA) for one hour. Cell nuclei were stained with NucBlue Fixed Cell Stain Ready Probes (Thermo Fisher Scientific, Waltham, MA). Neutral lipids were stained with Oil Red O. Before imaging, coverslips were mounted on glass slides with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA).

Western Blotting

Cells were lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA). Gel electrophoresis of the total protein samples were done in 4%-20% gradient precast gels (Mini Protean TGX gel, BioRad, Hercules, CA) and transferred to polyvinylidene fluoride (PVDF) membrane (BioRad, Hercules, CA) for 35 min. Membranes were incubated with primary antibodies, anti phospho-Akt (Ser 473), anti phospho-mTOR (Ser 2448) and anti phospho-ULK1 (Ser 757) to stain the phosphorylated form of these proteins (all from Cell Signaling Technology, Danvers, MA). Actin proteins were stained using beta-actin antibody (Mouse IgG2b, Cell Signaling Technology, Danvers, MA) for loading control.

Live cell imaging

Bone marrow macrophages were plated in 6 cm dishes and exposed to Ac-LDL and Bodipy C12 red 558/568 (1 μ M, Thermo Fisher Scientific, Eugene, Oregon, USA) for 24 h. Macrophages were incubated with regular BMM for another 6 and 24 h. For staining mitochondria and lysosomes, cells were incubated with Mitotracker green (100nM, ThermoFisher Scientific, Eugene, Oregon, USA) or Lyso Tracker green (50nM, ThermoFisher Scientific, Eugene, Oregon, USA) for 30 min prior to imaging. Images were acquired by an Andromeda spinning disk confocal microscope.

Electron microscopy

Bone marrow macrophages were exposed to Ac-LDL (50 μ g/ml) for 24 h. Medium was replaced by fresh bone marrow medium for 4, 24 and 48 h. Cells were centrifuged and fixed in 2% glutaraldehyde in 4% PFA. Cells were micro-centrifuged and post-fixed in 1% osmium tetroxide. Dehydration was done by using ethanol with increasing concentrations. After embedding in propylene oxide, polymerization was done at 60°C for overnight. Ultra-thin sections (50-70 nm) were made using an ultramicrotome.

Imaging

For fixed cells on the coverslips images were acquired using an EVOS FL imaging system containing GFP, CY5, DAPI filter cubes (ThermoFisher Scientific, Waltham, MA). An Andromeda spinning disk confocal microscope was used for the acquisition of live cell images. The 561nm laser with Zet 442/514/561x excitation wheel

and 605/64 emission wheel were used for “red.” The 445 lasers with Zet 442/514/561x excitation wheel and 537/26 emission wheel were used for “green.”

Image Analysis

ImageJ (National Institutes of Health, Bethesda, MA) (Schneider, Rasband et al. 2012) and Cell Profiler (Carpenter, Jones et al. 2006) were used to process images and quantify raw data. All raw images were visualized, cropped, and processed into montages using ImageJ. Data quantification was done using Cell Profiler. For measuring fluorescence intensity in cell profiler, a pipeline was constructed with different modules. For subtracting background, total image intensity was measured and lower quartile intensity was subtracted from the total image intensity. Nuclei were identified as primary objects using automatic threshold strategy and other proteins stained with antibody were identified as secondary objects using propagation method. The same proteins stained were used to train cell profiler to find the edge of the cell. Finally, object intensity was measured based on identified primary and secondary objects.

Statistical Analysis

Statistical analyses were done using GraphPad Prism software version 6 (GraphPad Software, Inc, La Jolla, CA). Values are given as mean fluorescence intensity ($n = 2-3$ coverslips/timepoint, 60-100 cells/coverslip). Absent error bars in the bar graphs signify the values smaller than the graphic symbols. Statistical analysis was carried out using one-way ANOVA and two-way ANOVA. Tukey’s multiple comparison test and Holm-Sidak’s test were performed for post hoc comparison of means. P-values less than 0.05 were considered significant.

RESULTS

Macrophage lipid droplets are degraded following Ac-LDL removal from culture medium

We exposed bone marrow macrophages to Ac-LDL for 24 h to load cells with lipid droplets. Then, Ac-LDL was removed and lipid droplet abundance was monitored in cells in growth medium for 4, 24 or 48 h (Fig. 1) to see if lipid droplets are stable in cells. Oil Red O was used to stain lipid droplets. Fluorescent microscopy images show that 4, 24 and 48 h after Ac-LDL removal from medium, the mean integrated intensity of Oil Red O decreased compared to 0 h ($p < 0.05$; Fig. 2). This result suggests that lipid droplets in lipid-laden macrophages are quickly degraded following a decrease in the cholesterol availability in the cellular environment.

Lipid droplet associated proteins appear as circular, ring-like structures surrounding lipid droplets.

Lipid droplets are ubiquitous organelles found in almost all organisms including humans, yeast, and bacteria. The surface of lipid droplets is decorated with many proteins some of which are shared by other organelles. Proteins exclusive to lipid droplets are known as PAT (perilipins, adipophilins and TIP47s or tail-interacting protein 47 KD) (Bickel, Tansey et al. 2009). Adipophilin deletions in *ApoE*^{-/-} mice caused reduced lipid droplet formation in macrophages which suggests that adipophilin is important for the formation of foam cells (Paul, Chang et al. 2008). To get some idea about adipophilin presence on lipid droplets during lipid droplet degradation, cellular adipophilin was

immunostained with anti-ADFP antibody and a fluorescent secondary antibody. After staining lipid droplets with Oil Red O, we found ring-like structures of adipophilin on the edges of lipid droplets (Fig 3). Pixel intensities from lines drawn on the image show adipophilin rings at early and late time points (Fig 3B-E). The disappearance of adipophilin rings correlates with the disappearance of lipid droplets (Fig 3A).

The autophagosomal marker protein, LC3, decreases within 24 h after Ac-LDL removal from medium.

The LC3 conjugation system is required for the formation of autophagosomes (Kabeya, Mizushima et al. 2000). Cytosolic LC3-I is conjugated with phosphatidylethanolamine (PE) to make the membrane bound form of LC3-II. LC3-II is located on pre-autophagosomes and autophagosomes after their formation and thus is used as a marker of autophagosomes (Kabeya, Mizushima et al. 2000). To investigate if autophagosomes are formed in foam cells after removal of Ac-LDL from the culture medium, we stained LC3 proteins with anti-LC3 antibody (Figure 4A). One-way ANOVA and Tukey's post hoc comparison show that following Ac-LDL removal from the culture medium, LC3 expression is decreased at 24 h compared to 0 h ($p < 0.05$; Fig. 4B).

Inhibition of the formation of autophagy machinery reduces lipid droplet clearance from macrophages.

In previous experiments, we found that lipid droplets are not permanent in foam cells. In the absence of Ac-LDL, lipid droplets are degraded starting within 24 h of LDL removal. If autophagy contributes to the metabolism of lipid droplets, then inhibiting the

formation of autophagosomes will prevent lipid droplet metabolism. Therefore, more lipid droplets are expected following removal of Ac-LDL from the culture medium if autophagosomes formation is inhibited. Autophagosome formation was inhibited by 3-methyl adenine (3MA), which inhibits the class III PI3K complex that is required for the formation of autophagosomes (Seglen and Gordon 1982). Lipid-laden macrophages were exposed to 3MA for 24-48 h after removal of Ac-LDL from the culture medium. Oil Red O staining of lipid droplets show that there are more lipid droplets at 48 h time point in 3MA inhibited cells compared to untreated cells (Fig 5A). The average fluorescence intensity of Oil Red O staining/cell was measured using Cell Profiler and graphed. Two-way ANOVA and Holm-Sidak post hoc comparison of means reveals that there is a greater average fluorescence intensity of 48 h, 3MA treated cells compared to control cells ($p < 0.05$) (Fig 5B).

Induction of autophagy does not affect lipid droplets disappearance from macrophages.

The role of autophagy in macrophage lipid metabolism demands more investigation. So, we used rapamycin, a pharmaceutical drug, to induce autophagy in macrophages and see if degradation of lipid droplets is hampered due to the induction of autophagy. Rapamycin is a drug that targets the mTOR pathway to inhibit growth signaling and promote autophagy. Lipid-laden bone marrow macrophages were treated with 5 nM rapamycin in bone marrow medium for 4, 24 or 48 h. Immunofluorescence staining of LC3 with anti-LC3 antibody shows the same fluorescence pattern as control cells with lipid droplet size and abundance decreasing over time indicating that promoting autophagy had no effect on the rate of lipid droplet metabolism (Fig. 6A).

Total fluorescence intensity per cell was measured by ImageJ. Two-way ANOVA and Holm-Sidak post hoc comparison of means shows that there is no significant difference of the fluorescence intensities between rapamycin treated and untreated cells, which suggests that induction of autophagy does not affect lipid droplet disappearance from macrophages (Fig 6B).

Inhibition of autophagy with 3MA during Ac-LDL treatment does not affect the formation of lipid droplets.

In fibroblast cells, previous work in fibroblasts found autophagy deposits fatty acids into lipid droplets (Rambold, Cohen et al. 2015). This raises questions whether autophagy has a role of autophagy in lipid droplet formation. The objective of this experiment was to see if lipid droplet formation is prevented in the absence of autophagy. Bone marrow macrophages were treated with 50ug/mL Ac-LDL for 4 and 24 with or without 3MA. Staining of lipid droplets with Oil Red O shows that there is no phenotypic difference between control and 3MA treated cells in lipid droplet accumulation. Immunofluorescence staining of LC3 was done as a marker of autophagosomes (Fig 7A). Total fluorescence integrated intensity was measured using CellProfiler. Even though quantified result shows a slight decrease of Oil Red O fluorescence in 3MA treated cells,

Two-way ANOVA and Holm-Sidak's post hoc comparison reveals that there is no significant difference between control and treated cells (Fig 7B).

Fatty acids localize to lysosomes but not mitochondria during lipid droplet degradation.

We designed a live-cell imaging experiment to stain fatty acids and mitochondria or lysosomes to see if lipid droplets are used as the source of β -oxidation or if they are going to lysosomes for cholesterol efflux. If lipid droplets were a source of fatty acids for beta oxidation in the mitochondria, then Bodipy C12 Red and Mitotracker would be co-localized during lipid droplet degradation. However, none was seen (Fig. 8). This result suggests that Ac-LDL induced lipid droplets are not utilized by mitochondria for beta oxidation. The result from lysosomal staining shows that before removing Ac-LDL from medium (0 h) fatty acids are in lysosomes (Fig 9B). This could be because fatty acids components of lipid droplets (triglycerides) trafficked through lysosomes prior to deposition in lipid droplets. Six hours following Ac-LDL removal, fatty acids have left lysosomes (Fig 9A) and after 24 h they are found in close proximity of lysosomes (Fig 9C). Fatty acids look surrounded by lysosomes and this may provide evidence that fatty acids traffic through lysosomes during lipid droplet formation as well as degradation. Furthermore, the close proximity of fatty acids and lysosomes during lipid droplet

degradation provides evidence to support the role of autophagy in the degradation of lipid droplets.

Phosphorylation of Akt is decreased during the formation and degradation of lipid droplets.

Since autophagy starts when cells are lipid loaded, we were interested to see if exposure to Ac-LDL or presence of lipid droplets changes growth-factor signaling in macrophages. Akt is a protein required for the growth of cells which activates the mTOR signaling pathway. Phosphorylated mTOR inhibits autophagy by phosphorylating and inhibiting unc-51-like kinase 1 (ULK1). Lipid-laden macrophages were immunostained for activated Akt (pAkt) during the formation of lipid droplets and 4 h following the removal of Ac-LDL from the culture medium (Fig 10A). Phosphorylation of Akt was lower in Ac-LDL cells compared to untreated cells at the -4, 0 and 4-h timepoints ($p < 0.05$, one-way ANOVA and Holm-Sidak's post hoc comparison of means) (Fig 10B). To further clarify this, we immunoblotted for pAkt, pmTOR and pULK1 to see how this pathway is affected by lipid droplet formation in macrophages. Immunoblot result shows slight different result from immunofluorescence staining of pAkt (Fig. 11). pAkt signal was reduced after the formation of lipid droplets in macrophages. On the other hand, pmTOR signal increases after Ac-LDL treatment which decreases after Ac-LDL removal (Fig 11). Similarly, pULK1 is also increased after Ac-LDL treatment as well, but start to decrease with the formation of lipid droplets (Fig 11).

Transmission electron microscopy shows double membrane organelles surrounding lipid droplets after formation of lipid droplets and 4 h after Ac-LDL removal from macrophages.

Bone marrow macrophages were treated with AC-LDL for 24 h and replaced with bone marrow medium for 0, 4 and 24 h and fixed. This was done to look for double membrane organelles surrounding lipid droplets to make sure about autophagy. In electron microscopy images, double membrane structure was found surrounding lipid droplets after formation of foam cells (0 h) and within 4 h of AC-LDL removal from culture medium (Fig 12).

DISCUSSION

Here we present evidence that autophagy facilitates the degradation of lipid droplets in Ac-LDL exposed macrophages. Lipid droplets made following Ac-LDL exposure are quickly degraded in macrophages after withdrawal of LDL. However, this degradation is inhibited in the presence of the PI3K inhibitor 3-MA. Furthermore, double lipid bilayers are seen on lipid droplets in macrophages and live-cell microscopy shows trafficking of fatty acids to lysosomes during lipid droplet degradation. Akt phosphorylation is decreased in lipid-laden macrophages compared to controls. Lowered activation of Akt may be a mechanism for the induction of autophagy in lipid-laden macrophages.

In exploring lipid droplet degradation in macrophages, we found that after formation of foam cells, lipid droplets get digested in the absence of Ac-LDL in the culture medium. Previous study also shows that autophagy is activated after lipid loading

in macrophages (Ouimet, Franklin et al. 2011). From this point of view, we stained LC3II. LC3II is extensively used as a marker of autophagosomes. Accumulation of LC3II is one line of evidence supporting autophagy. Lipid droplet stained with Bodipy C12 Red were also found near lysosomes which correlates with the idea of lysosomes docking to lipid droplets during lipid droplet degradation through autophagy in hepatocytes (Schroeder, Schulze et al. 2015).

Reduced pAkt and pULK1 signal is another evidence supporting the idea of autophagy induction after foam cell formation. Increased pmTOR signal during the formation of lipid droplets is somewhat confusing which may indicate that autophagy is activated in a mTOR independent pathway. When mTOR is activated or phosphorylated, it inhibits autophagy by phosphorylating ULK1. The presence of pULK1 means it's inhibited. On the other hand, absence of pULK1 means ULK1 as well as autophagy is active. We got reduced signal of pULK1 at 4 h which indicates that autophagy is active even though mTOR is activated at the same time. This will be interesting to investigate how pULK1 is reduced in the presence activated mTOR and if inhibition of Akt phosphorylation reduces lipid droplets get degradation or not.

In conclusion, Ac-LDL treatment or lipid droplet formation induces autophagy in lipid-laden macrophages through inhibition of phosphorylation of Akt and activation of ULK1. Autophagy then delivers lipid droplets to lysosomes for degradation. However, autophagy is not important for the formation of lipid droplets in macrophages.

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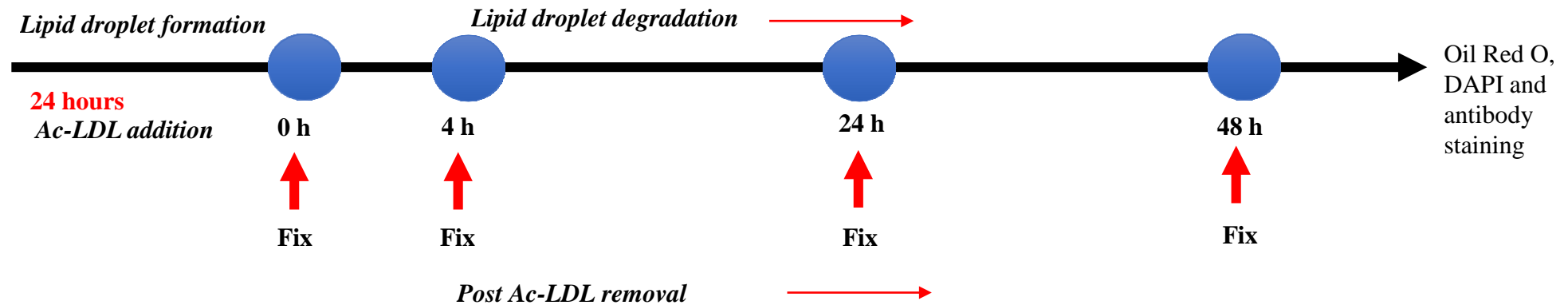


Figure 1. Experimental timeline of Ac-LDL induced formation of lipid-laden macrophages and lipid droplet degradation. Lipid-laden macrophages were made by treating bone marrow or fetal liver macrophages with acetylated low-density lipoproteins (Ac-LDL) for 24 h. To follow their degradation, Ac-LDL media was replaced with fresh media and incubated for 0, 4, 24 and 48 h and fixed.

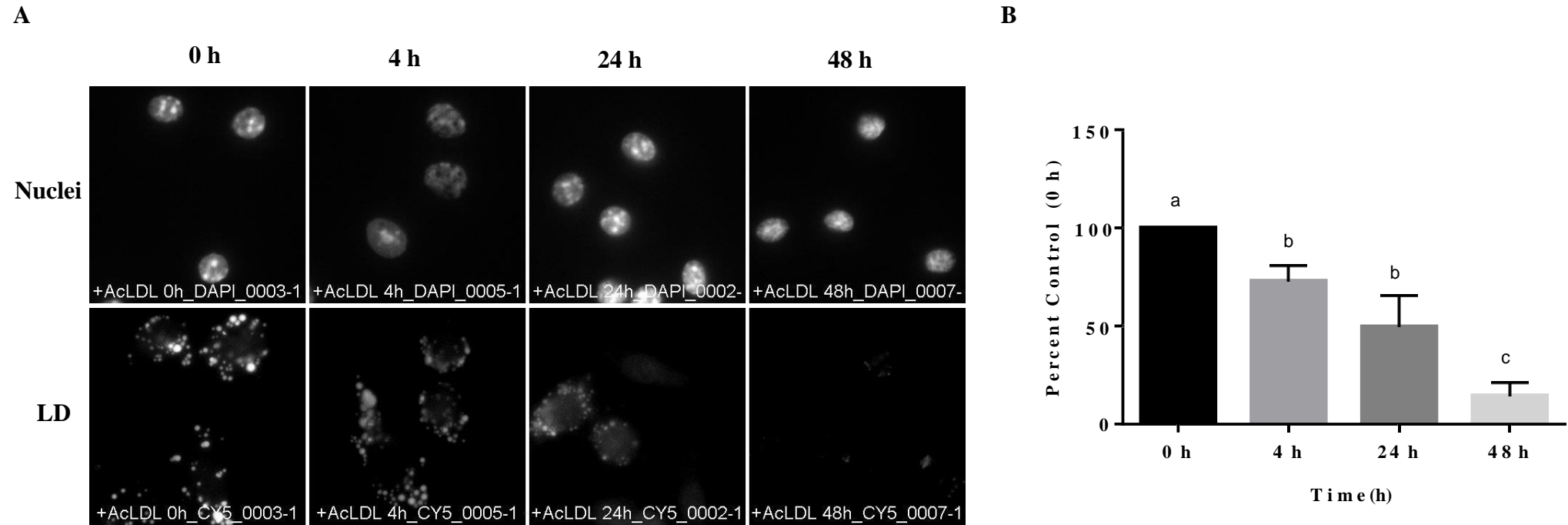


Figure 2. Macrophage lipid droplets are degraded following Ac-LDL removal from culture medium. Bone marrow macrophages were loaded with lipid droplets by culturing with Ac-LDL for 24 h. Ac-LDL medium was replaced with bone marrow media when all the cells were filled up with lipid droplets. Cells were incubated with bone marrow medium for 4, 24 and 48 h and then fixed. A) Images from epifluorescence microscope show lipid droplets labelled with Oil Red O and nuclei labelled with DAPI, B) Total fluorescence intensity of Oil Red O was measured using Image J and graphed. Error bars show standard deviations. Different letters represent statistical differences, $p < 0.05$. $n = 3$ coverslips/timepoint (~100 cells/coverslips). LD = Lipid droplets.

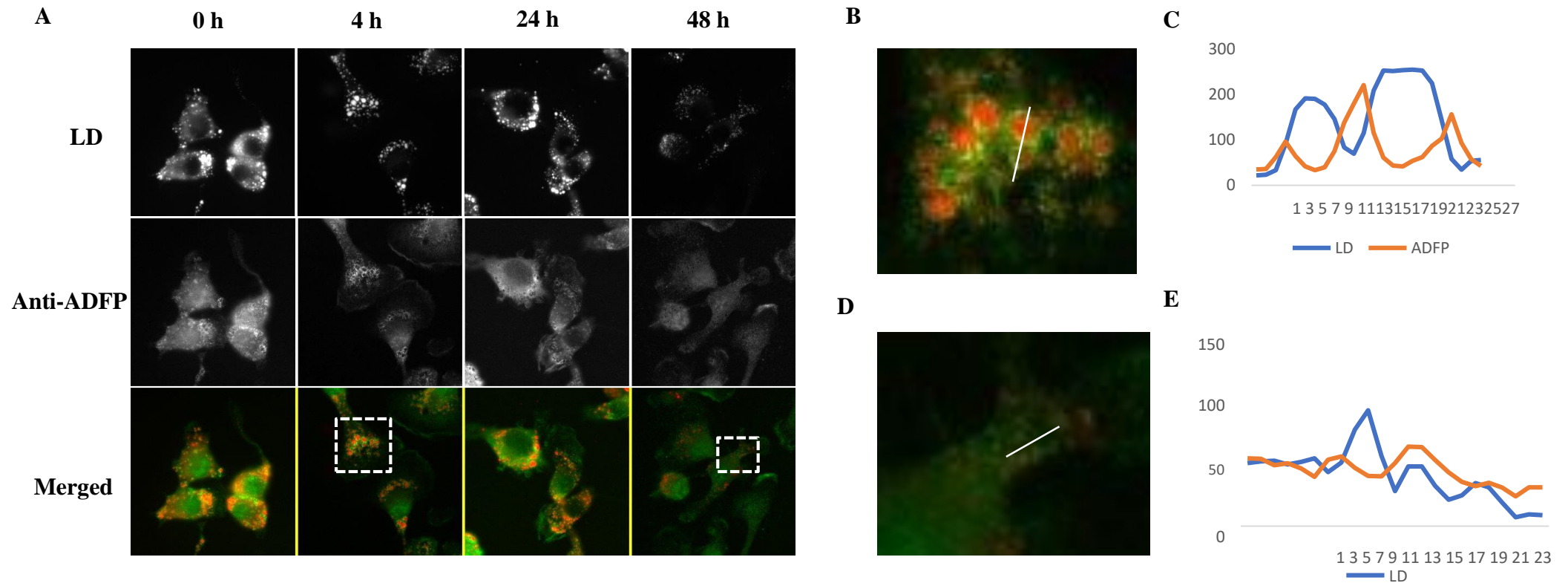


Figure 3. Adipophilins form ring-like structure surrounding lipid droplets and remain associated with them during metabolism. Lipid droplet associated protein, adipophilin, was stained with anti-ADFP antibody. A) Immunofluorescence staining of ADFP showing the ring structures surrounding lipid droplets. Images were taken from inverted microscope with oil objective, B) Inset from A at 4 h (white box), C) Gray value/pixel intensity was measured along the line in lipid droplets and ADFP images and graphed, D) Inset from A at 48 h (white box), E) Gray value/pixel intensity was measured along the line in lipid droplets and ADFP images and graphed. LD = Lipid droplets.

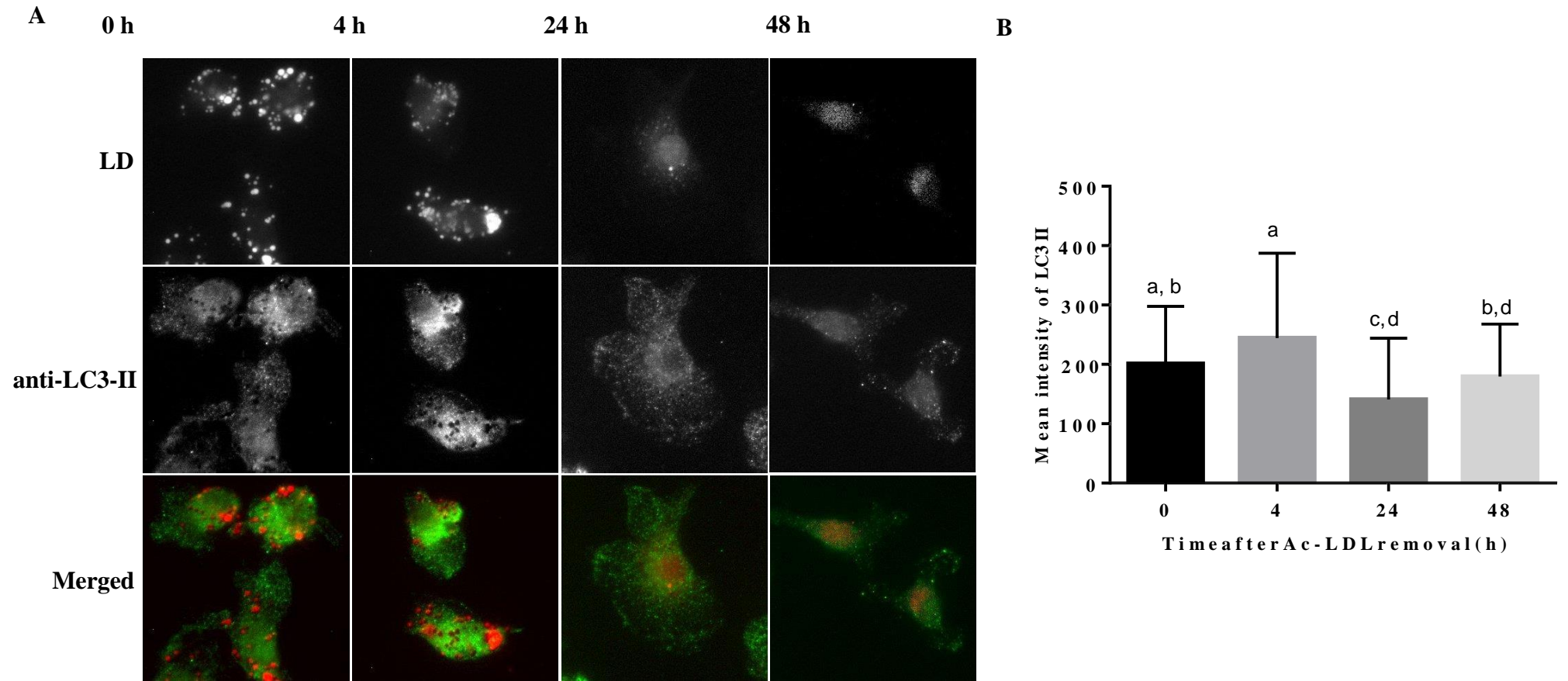


Figure 4. LC3-II decreases within 24 h after Ac-LDL removal from culture medium. Cells were fixed at 0, 4, 24 and 48 h following Ac-LDL removal from medium. Images were taken by epifluorescence microscope. A) Immunostaining of LC3-II (green) and Oil Red O staining of lipid droplets (red), B) Mean fluorescence intensity of LC3-II. Error bars show standard deviations. LD = Lipid droplets. Different letters represent statistical difference $p < 0.05$. $n = 3$ coverslips/timepoint (~100 cells/coverslips)

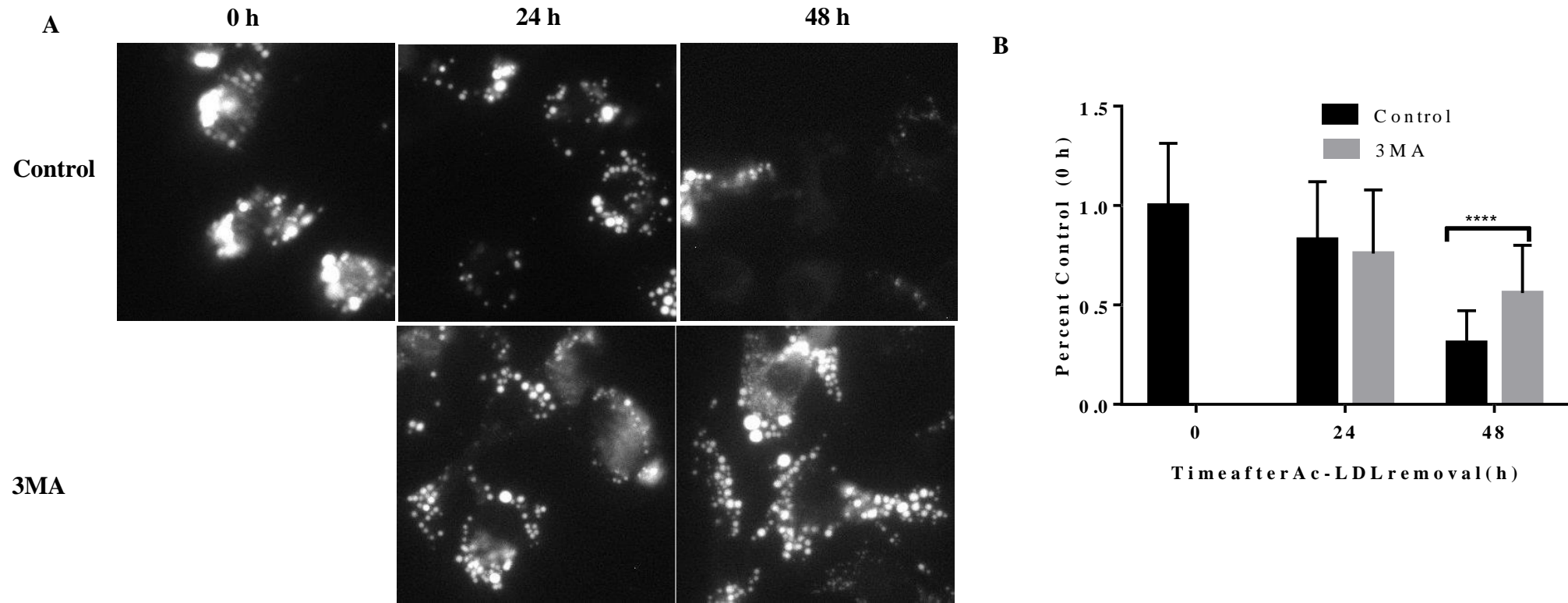


Figure 5. Cells have more lipid droplets after 48 h of 3MA treatment compared to control cells.

A) Lipid droplets were stained with Oil Red O in control and 3MA treated lipid-laden macrophages following Ac-LDL removal from the culture medium. B-D) Integrated fluorescence intensity of lipid droplet fluorescence was measured using Cell Profiler and graphed as percent 0-h control cells. Error bars represent standard deviations. N= 2-3 fields of view (60 cells) B) Experiment 1, C) Experiment 2 and D) Averages from two experiments. **** $p \leq .0001$, *** $p \leq .001$, ** $p \leq .01$, * $p \leq .05$

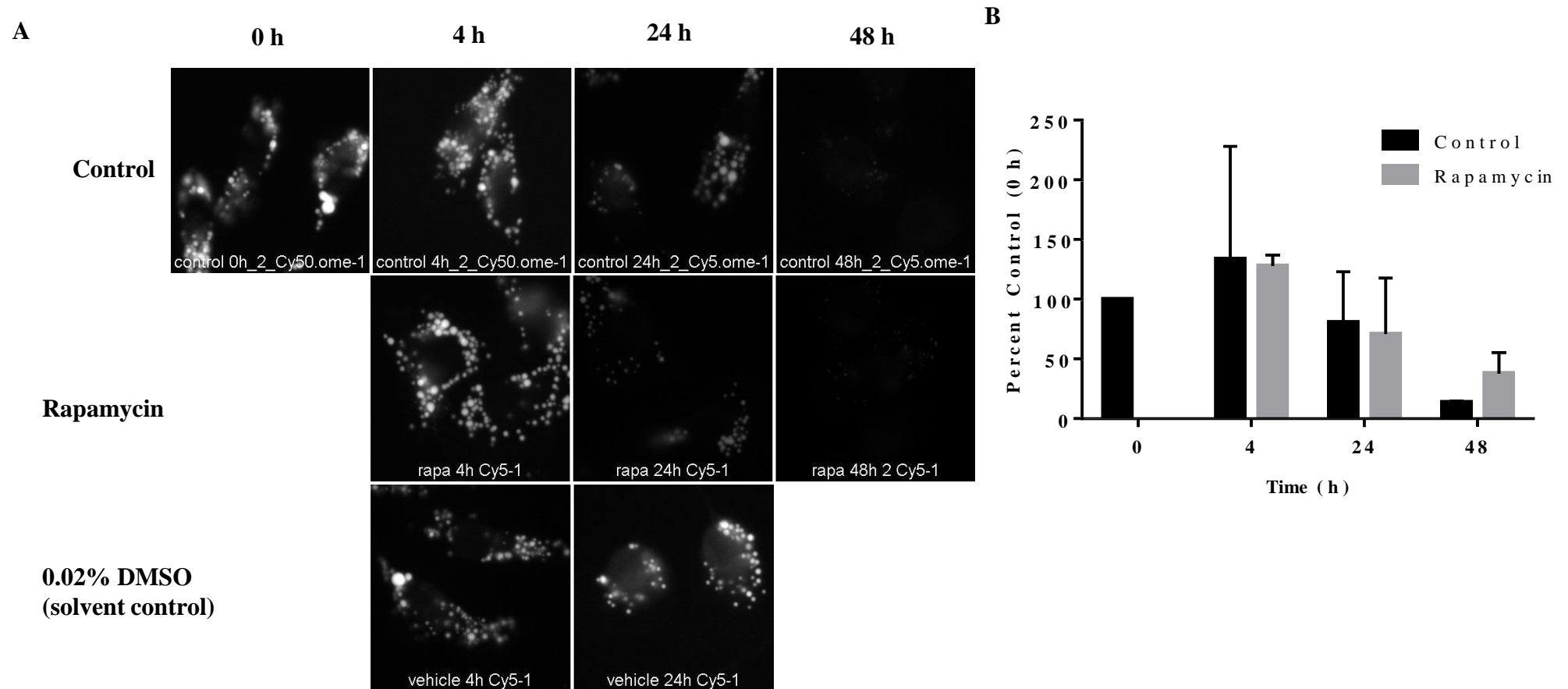


Figure 6. Induction of autophagy does not prevent lipid droplet disappearance from cells.

Cells were treated (w/wo Rapamycin) for 0, 4, 24 and 48 h after Ac-LDL removal. A) Images from inverted microscope show lipid droplets stained with Oil Red O. B) Mean fluorescence intensity of Oil Red O was measured in Cell Profiler and graphed. Error bars are showing standard deviations. N= 2 coverslips (~100 cells/coverslip).

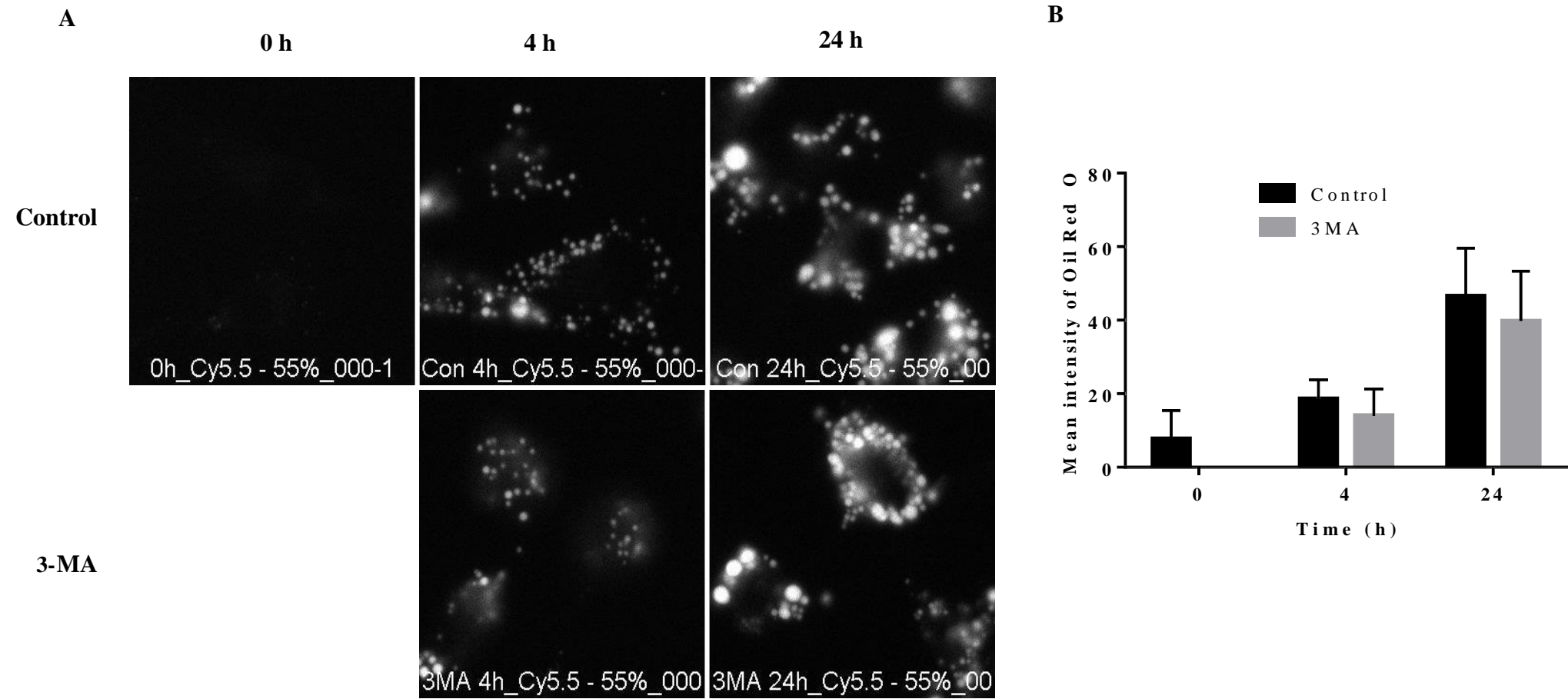


Figure 7. Inhibition of autophagy by 3MA does not affect the formation of lipid droplets.

Cells were treated (w/wo 3MA) for 4 and 24 h during the treatment with Ac-LDL. A) Images from fluorescence microscope show lipid droplets stained with Oil red O. B) Mean fluorescence intensity of Oil Red O was measured in Cell Profiler and averages of two independent experiments are graphed. Error bars show standard deviation. N= 2 coverslips (~100 cells/coverslip).

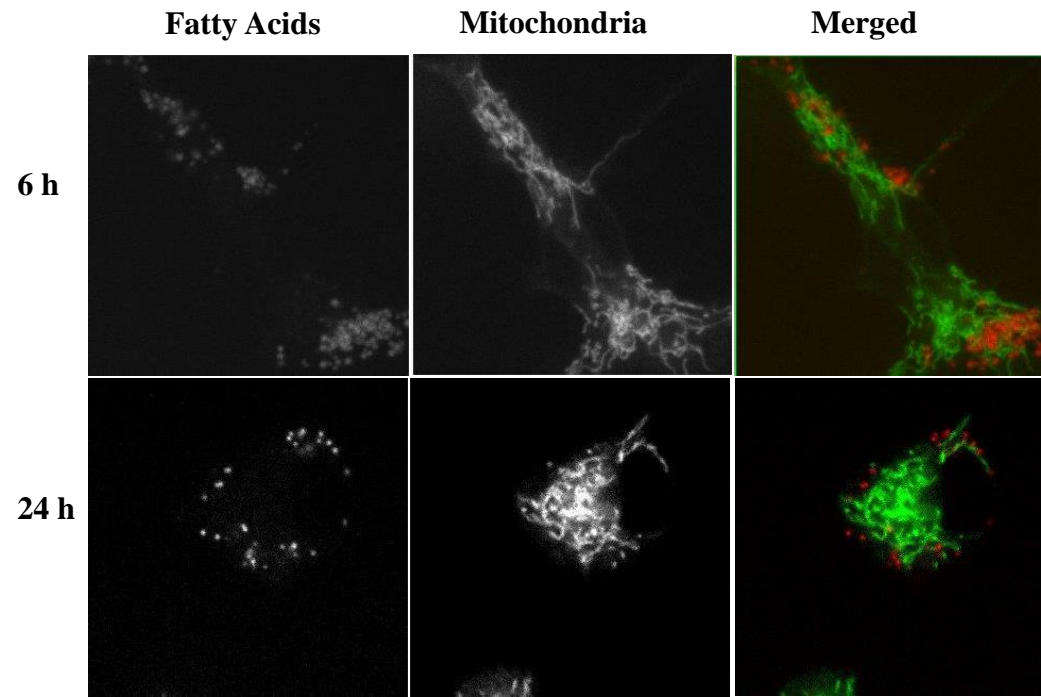


Figure 8. Fatty acids do not localize in mitochondria after 24 h.

Bone marrow macrophages were incubated with Ac-LDL and BODIPY C12 Red (a marker for fatty acids) for 24 h. Cells were incubated for another 1 h with bone marrow media so that all the C12 red enters lipid droplets. Then cells were incubated with complete media for 6 and 24 h and imaged by spinning disk confocal microscope to determine subcellular localization of fatty acids. Mitochondria were labelled with Mito Tracker Green. FA = Fatty acids.

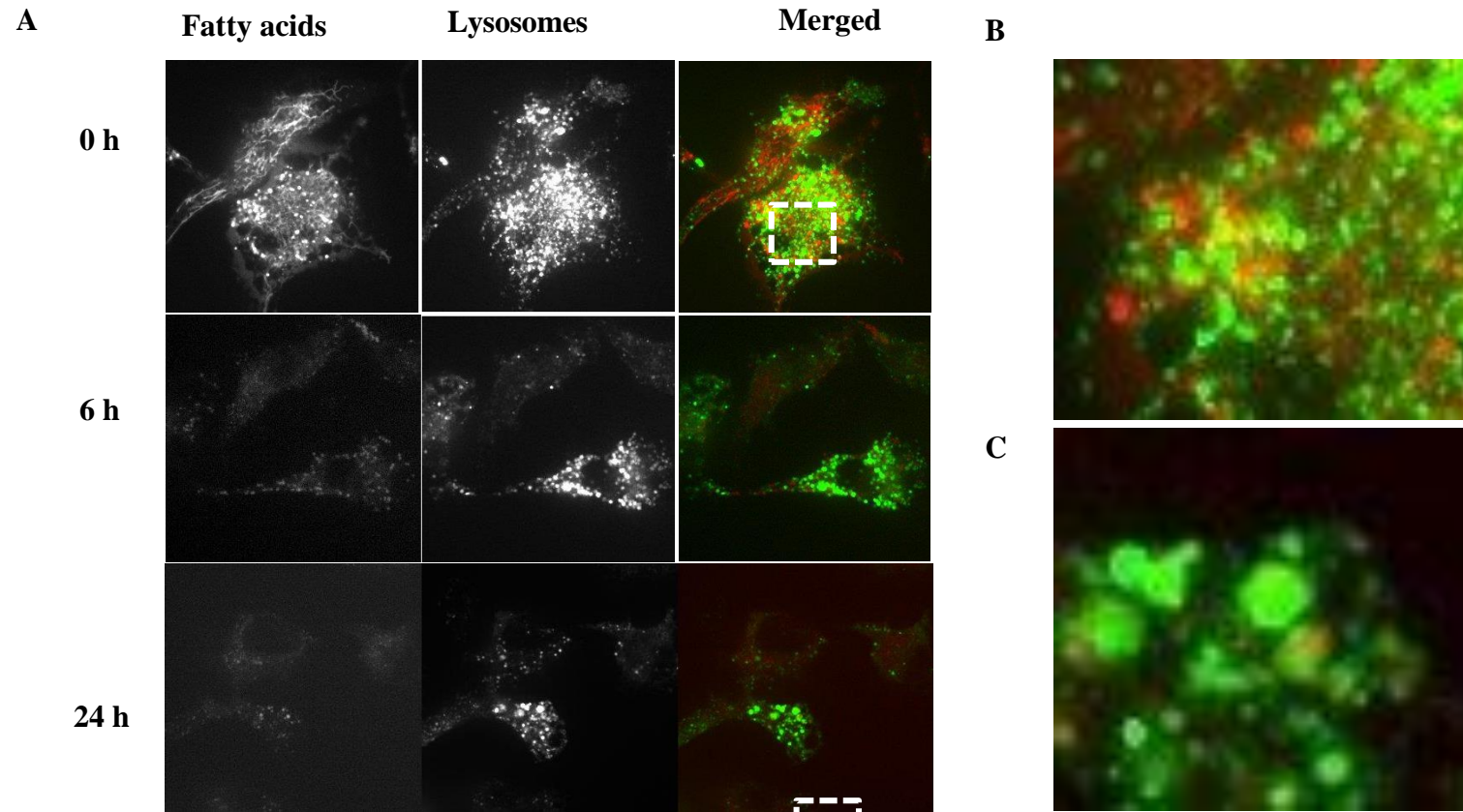


Figure 9. Fatty acids do not localize in lysosomes after 24 h of Ac-LDL removal from macrophages.

Bone marrow macrophages were incubated with Ac-LDL and BODIPY C12 Red for 24 h. Cells were incubated for another 1 hour with complete media so that all the C12 red enters lipid droplets. A) Then cells were incubated with bone marrow media for 6 and 24 h and imaged by spinning disk confocal microscope to determine subcellular localization of fatty acids. Lysosomes were labelled with Lyso Tracker Green. B) Inset of A from 0 h row (white box), C) Inset of A from 24 h row (white box). FA = Fatty acids.

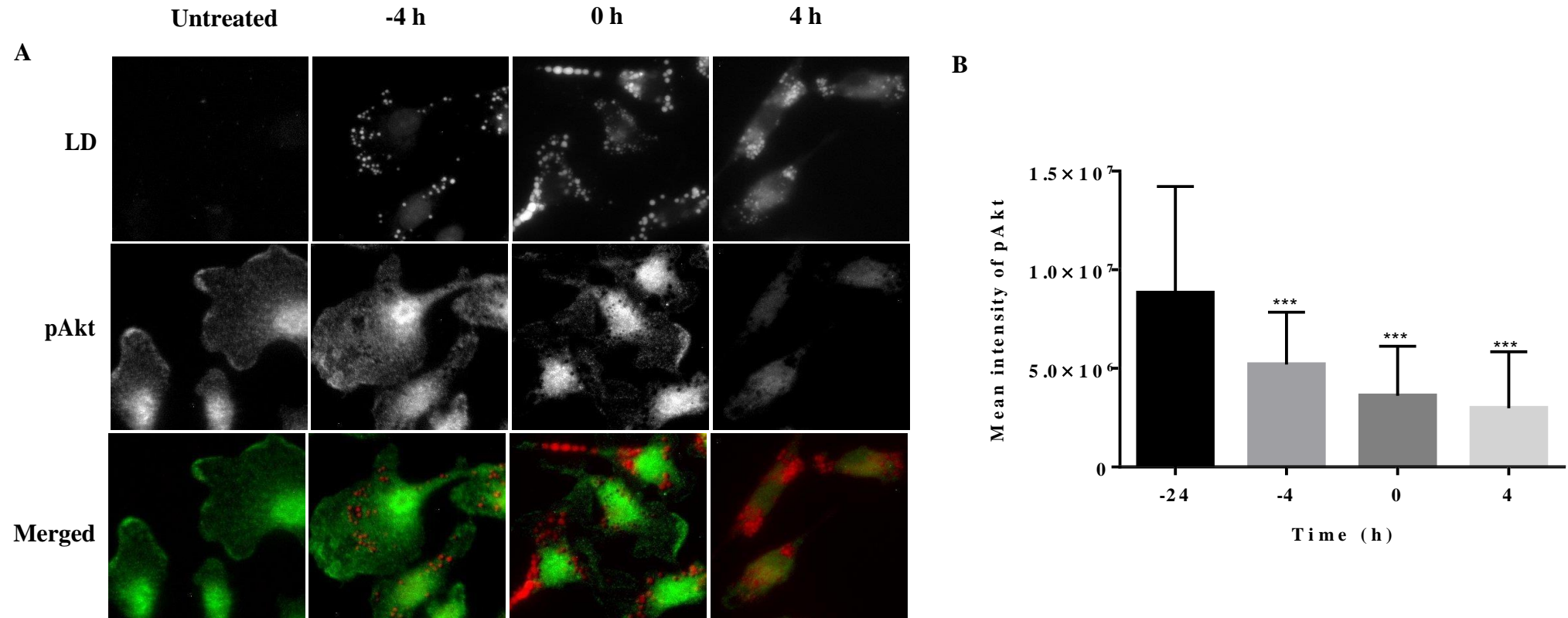


Figure 10. pAkt decreases during Ac-LDL treatment, formation of lipid droplets, and during their degradation.

Bone marrow macrophages were treated with Ac-LDL (50 $\mu\text{g/mL}$) for 4 and 24 h and fixed. For some cells Ac-LDL media was replaced by fresh bone marrow media for another 4 h and fixed. Immunofluorescence staining of pAkt was done using anti-pAkt primary antibody. Lipid droplets were stained with Oil Red O. B) Mean fluorescence intensity of pAkt was measured using image j and graphed. Different letters are representing statistical differences. Error bars are showing standard deviations. N= 1 coverslips (40 cells)

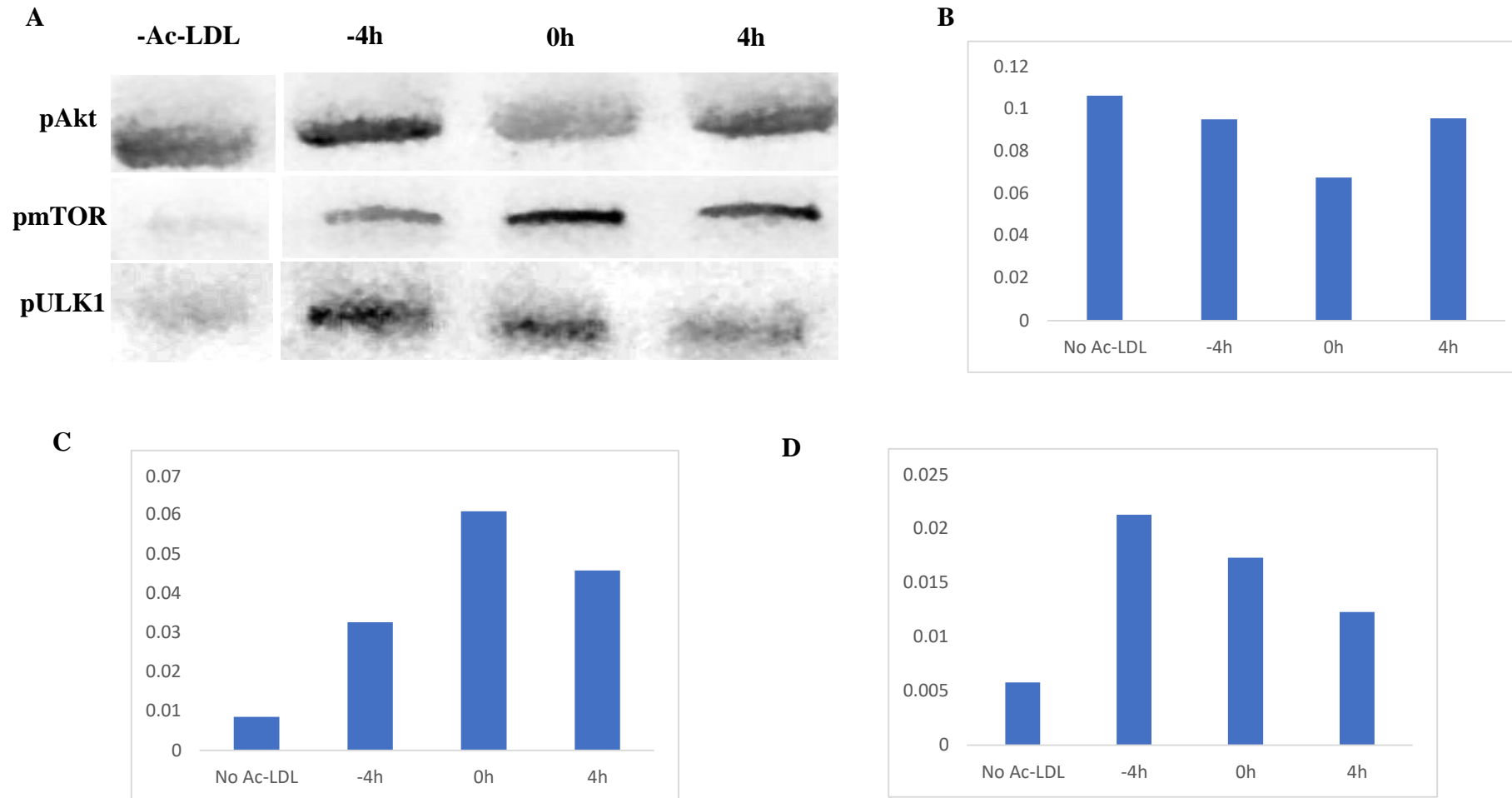


Figure 11. Western blot with three important proteins in autophagy signaling pathway.

A) Western blot images of pAkt, pmTOR and pULK1, B) Quantification of pAkt band intensity, C) pmTOR band intensity, D) pULK1 band intensity.

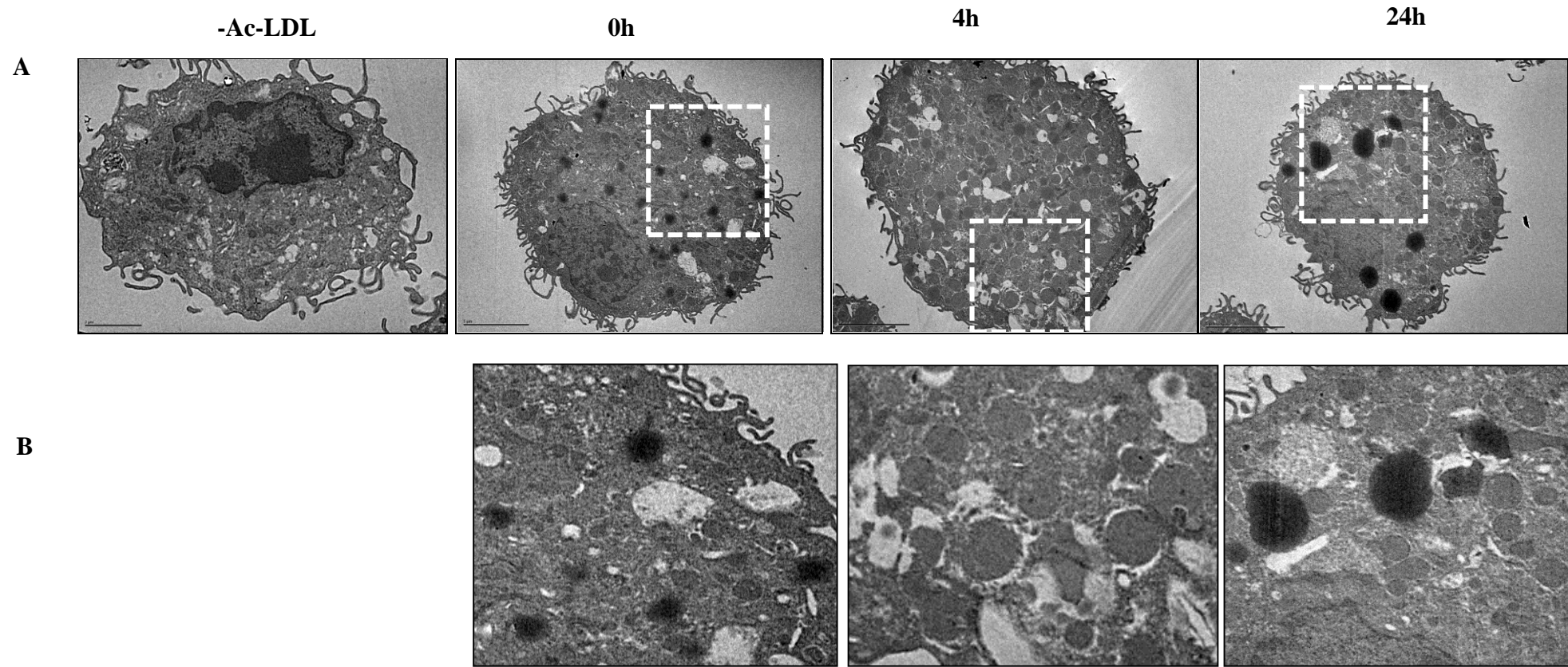


Figure 12. Transmission electron microscope images.

A) Cells at different timepoints of lipid droplet formation and degradation, B) Insets from A (white boxes).

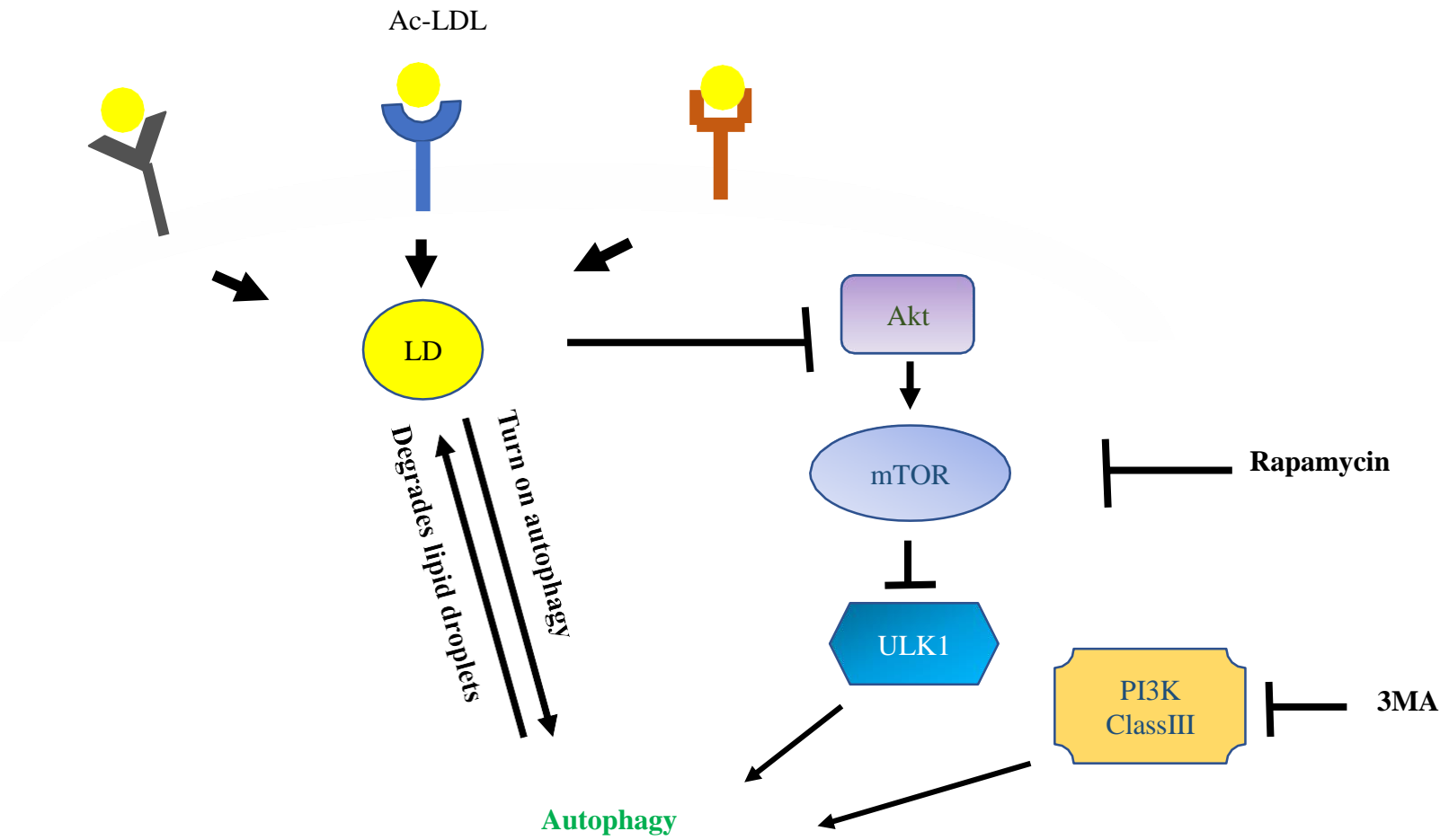


Figure 13. Conclusion

CHAPTER THREE

CONCLUSION

Lipid-laden macrophages are the key mediators of the plaque formation in atherosclerosis. Macrophages become lipid-laden by internalizing modified low-density lipoproteins and storing them in lipid droplets. They are known as foam cells because of their foamy appearance viewed by microscope. In macrophages, neither the mechanisms of lipid droplet formation nor the mechanisms of lipid droplet degradation are well understood. It is important to understand these basic processes to identify therapeutic targets for the treatment of atherosclerosis. The goal of this project was to understand the role of autophagy in lipid droplet clearance from macrophages. Investigation into signaling and trafficking mechanisms of autophagic clearance from macrophage lipid droplets will help us to design therapeutics to treat atherosclerosis.

Autophagy is a cellular process used for degrading cellular components for energy production which is found to play an important role in the degradation of lipid droplets in hepatocytes (Schroeder, Schulze et al. 2015). In this process, autophagosomes are formed surrounding cellular components for delivery to lysosomes for degradation. However, how this process is activated in macrophages following lipid droplet formation is not clearly understood. Similarly, how lipid droplets signal for autophagy and which proteins are involved in this process still needs to be investigated. Furthermore, it is important to study lipid droplet associated proteins to see if any of them are part of

autophagy signaling process. Studying adipophilin will be a good start to look for the lipid droplet associated proteins that facilitate lipid droplet degradation in macrophages.

Following lipid droplet formation in macrophages, autophagy quickly degrades lipid droplets. Lipid droplet metabolism can be prevented by the inhibition of autophagosome formation. We found the lipid droplets in macrophages are not used as a source of β -oxidation, rather they are degraded by lysosomes for cholesterol efflux. There is also some effect of lipid droplet formation on phosphorylation of Akt. Exposure to acetylated LDL or the presence of lipid droplets may cause inhibition of phosphorylation of Akt or may increase the rate of Akt dephosphorylation by phosphatases. Increased mTOR signal in the presence of decreased pAkt signal may suggest that this activation of autophagy is mTOR independent (Fig 13).

In this study, acetylated LDL was used to form lipid droplets since it is processed quickly. However, it is not found in vivo and there are some debates how its metabolism in cell differ. It will be worthwhile to perform these experiments with oxidized LDL instead of acetylated to see if treatment of oxidized LDL or the presence of lipid droplets triggers autophagy in the same that Acetylated LDL does.

To further study signaling events, inhibiting the Akt signaling pathway and monitoring lipid droplet degradation will be worthwhile to see if Akt has any effect on lipid droplet degradation as well as autophagy.

Understanding the mechanism of lipid droplet metabolism and underlying signaling events will bring the novel therapeutic strategies that reduce the lipid burden in atherosclerotic plaques. The data I collected will be helpful to further research efforts

leading to developing therapeutic strategies targeting foam cell macrophages to treat atherosclerosis.

References

Schroeder, B., R. J. Schulze, S. G. Weller, A. C. Sletten, C. A. Casey and M. A. McNiven (2015). "The small GTPase Rab7 as a central regulator of hepatocellular lipophagy." Hepatology **61**.